



# TRANSLATOR CERTIFICATION

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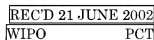
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This is to certify that the annexed is a true copy of the following application as filed with this office.

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[Title of the Invention]

[Claims]

[Claim 1] Any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) ) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 2] Any one of the following DNAs:

- (1) a DNA having the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 3] Any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 4] Any one of the following DNAs:

- (1) a DNA having the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[Claim 5] A DNA encoding any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 6] A DNA encoding any one of the following proteins:

- (1) a protein having the 84<sup>th</sup> residue ~ 804<sup>th</sup> residue of the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the 84<sup>th</sup> residue ~ 804<sup>th</sup> residue of the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 7] The DNA according to any one of Claims 1-6, wherein a cytoplasmic male sterile individual organism has cytoplasmic male sterile genes of kosena radish and/or ogura radish or their homologues.

[Claim 8] Any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 9] Any one of the following proteins:

- (1) a protein having the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue of the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue of the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[Claim 10] The protein according to claim 8 or 9, wherein a cytoplasmic male sterile individual organism has cytoplasmic male sterile genes of kosenia radish and/or ogura radish or their homologues.

[Claim 11] A vector carrying a DNA according to any one of claims 1-7.

[Claim 12] A transformant having a DNA according to any one of claims 1-7 or the vector according to claim 11.

[Claim 13] The transformant according to claim 12, said transformant being a transgenic plant.

[Claim 14] A method for restoring fertility from a cytoplasmic male sterile individual organism, wherein a DNA according to any one of claims 1-7 is used.

[Claim 15] A transformant that allows controlling the expression of a male fertility restorer gene by introducing part or whole of a DNA according to any one of claims 1-7 together with an inducible promoter into a cell having both a cytoplasmic male sterile gene and a DNA according to any one of claims 1-7.

[Claim 16] A method for maintaining a cytoplasmic male sterile line using the transformant according to claim 15.

[Claim 17] A method for detecting a gene involving in the restoration of cytoplasmic male fertility by using an oligonucleotide primer of 15-mer ~ 50-mer optionally set from a DNA according to any one of claims 1-7 or a probe of at least 15-mer or more consisting of part or whole of a DNA according to any one of claims 1-7 and confirming that the amount of base sequence amplified by the primer or the amount of base sequence detected by the probe is one gene or more in one genome of a biological sample.

[Detailed Explanation of the Invention]

[0001]

[Industrial Field of Application]

The present invention relates to a gene involving in the restoration of fertility from cytoplasmic male sterility. More specifically, the invention relates to a gene involving in the restoration of fertility from cytoplasmic male sterile traits, which may hereinafter be referred to as cms, used for developing the first filial hybrid (hereinafter referred to as F<sub>1</sub>), a vector containing the aforementioned gene and a transformant.

[0002]

[Prior Art]

The development of F<sub>1</sub> strains has been popular and widely used in practice for a wide range of main agricultural products such as cereal crops and vegetables in consideration of (1) excellent agronomic traits by hybrid vigor, (2) the uniformity of crops and (3) the protection of the benefits of breeders because acquired characteristics can be separated in the next generation.

[0003]

Methods for producing the seeds of F<sub>1</sub> strains include a cms – Rf seed growing system using cytoplasmic male sterility (cms) lines and male fertility restoration (which may hereinafter referred to as Rf) lines, which has been used for cereal crops such as rice, sorghum and corns and oil crops such as sunflowers using a crossbreeding or cell fusion method.

[0004]

On the other hand, an F<sub>1</sub> seed growing system using self incompatibility has been widely used for cruciferous vegetables. As far as rapeseeds are concerned, however, an F<sub>1</sub> seed growing system using cms lines and Rf lines has been used because there is no stable self incompatibility.

[0005]

Much research has recently been conducted for rapeseeds using cytoplasmic male sterility derived from konase radish (konase cms) and cytoplasmic male sterility derived from ogura radish (ogura cms). Both cms genes are encoded in genomes of mitochondria, a cytoplasmic organelle, and their base sequences are known. However, radish lags behind other plants in molecular-biological research, and few markers necessary for isolating genes are known. For this reason, it is difficult to isolate genes from a nucleus, and therefore Rf has been introduced into rapeseeds using a sterility restoration line of radish by a crossbreeding or cell fusion method.

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[0006]

As far as Rf genes are concerned, there are one or more restorer genes depending on the cms lines of plants. It has been known that the presence of both Rf1 and Rf2 are necessary for the restoration of fertility in radish, and that the Rf1 gene significantly decreases the amount of accumulated ORF125 protein (M. Iwabuchi et al. Plant Mol. Biol. 39:183-188, 1999) in the mitochondria, which is known to be the protein causing cms of radish (Journal of Thremmatology 47 (additional volume 1) P186, 1997, Journal of Thremmatology 48 (additional volume 1) P197, 1998).

[0007]

In the rapeseeds, it has been known by genetic analysis experiments that the Rf1 gene of radish introduced by crossbreeding or cell fusion decreases the amount of accumulated ORF125 or ORF138 protein, which is known as protein causing cms (M. Grelon et al. Mol. Gen. Genet. 243:540-547), and that the phenomenon of a decrease in the amount of accumulated ORF125 or ORF138 protein completely agrees with the phenomenon of the restoration of fertility (N. Koizuka, et al. Theor Appl Genet, 100:949-955, 2000). In other words, the restoration of fertility requires a decrease in the amount of accumulated ORF125 or ORF138 protein in the rapeseed male sterility line. In this respect, the Rf1 gene is a significant gene.

Nevertheless, while the base sequence of an Rf2 gene, a restorer gene for T-cytoplasm that is a cms of corns, was identified and isolated, the base sequences of Rf genes have not been known yet in the other plants.

[0008]

[Problems that the Invention is to Solve]

It has been known that the content of glucosinolate (hereinafter "GSL") becomes higher than the regulation value in F<sub>1</sub> strains made by a rapeseed restoration line introduced with a Rf1 gene by crossbreeding or cell fusion and the same line as a father, which is a problem in terms of practical use. It is believed that the content of GSL increases in the restoration line (Rf line) of rapeseeds because a radish-derived gene involving in the biosynthesis of GSL exists in the neighborhood of the Rf1 gene, and therefore they are strongly linked genetically. GSL is contained in the pressed oil cake of rapeseeds and known to cause hyperthyroid when animals are fed with it. The GSL content of rapeseeds should therefore be 18μ mole/g in North America and 20μ mole/g in Europe on the stage of breeding.

[0009]

Furthermore, plants added with the function of herbicide resistance by gene splicing have recently been developed on a wide scale. In order to create these plants efficiently, it is insufficient to use only the rapeseed restoration line obtained by crossbreeding or cell fusion. The isolation of Rf genes, especially radish-derived Rf1 genes, has strongly been desired.

[0010]

The object of the present invention is to isolate an Rf gene, especially a radish-derived Rf1 gene, and identify its structure. Moreover, the invention is to provide a means of establishing the rapeseed restoration line using the isolated Rf gene.

[0011]

[Means of Solving the Problems]

The present inventors committed themselves to solving the aforementioned problems and succeeded in cloning an Rf1 gene from radish. Thus, they could solve the aforementioned problems.

In other words, the present invention provides any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

The present invention also provides any one of the following DNAs:

- (1) a DNA having the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 and involves in the restoration of fertility from cytoplasmic male sterility.

[0012]

The present invention also provides any one of the following DNAs:

- (1) a DNA having the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[0013]

The present invention also provides any one of the following DNAs:

- (1) a DNA having the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) a DNA that can hybridize with a DNA having the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 and involves in the restoration of fertility from cytoplasmic male sterility.

[0014]

The present invention also provides a DNA encoding any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0015]

The present invention also provides a DNA encoding any one of the following proteins:

- (1) a protein having the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

In the present invention, a cytoplasmic male sterile individual organism preferably has cytoplasmic male sterile genes of kosena radish and/or ogura radish or their homologues.

[0016]

The present invention also provides any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0017]

The present invention also provides any one of the following proteins:

- (1) a protein having the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

In the present invention, a cytoplasmic male sterile individual organism preferably has cytoplasmic male sterile genes of kosena radish and/or ogura radish or their homologues.

[0018]

The present invention also provides a vector containing a DNA according to the present invention.

The present invention also provides a transformant having a DNA according to the present invention or a vector according to the present invention.

The present invention also provides a method for restoring fertility from a cytoplasmic male sterile individual organism using a DNA according to the present invention.

The present invention also provides a transformant that allows controlling the expression of a male fertility restorer gene by introducing part or whole of a DNA according to the present invention together with an inducible promoter into a cell having both a cytoplasmic male sterile gene and a DNA according to the present invention.

The present invention also provides a method for maintaining a cytoplasmic male sterile line using the transformant according to present invention.

[0019]

Furthermore, the present invention also provides a method for detecting a gene involving in the restoration of cytoplasmic male fertility by using an oligonucleotide primer of 15-mer ~ 50-mer optionally set from a DNA according to the present invention or a probe of at least 15-mer or more consisting of part or whole of a DNA according to the present invention and confirming that the amount of base sequence amplified by the primer or the amount of base sequence detected by the probe is one gene or more in one genome of a biological sample.

[0020]

[Mode for Implementing the Invention]

A description of a mode for implementing the present invention is given below in detail.

(1) Mode of DNA according to the invention

The DNA according to the present invention relates to any one of the following DNAs.

- (1) a DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2, the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 or the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2;
- (2) a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2, the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 or the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 and involving in the restoration of fertility from cytoplasmic male sterility; or
- (3) ) a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1, SEQ ID NO: 2, the 3754<sup>th</sup> ~ 8553<sup>rd</sup> base sequence of the base sequence described in SEQ ID NO: 1 or the 250<sup>th</sup> ~ 2415<sup>th</sup> base sequence of the base sequence described in SEQ ID NO: 2 under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility.

[0021]

Moreover, the DNA according to the present invention relates to a DNA encoding any one of the following proteins.

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3, or the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 or the amino acid sequence of the 84<sup>th</sup> residue ~ the 804<sup>th</sup> residue described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0022]

As used herein, DNA according to the present invention may also be referred to as genes according to DNA.

The base sequence as shown in SEQ ID NO: 1 is a genomic DNA base sequence consisting of 8553 bases. The base sequence as shown in SEQ ID NO: 2 is a coding sequence assumed by SEQ ID NO: 1. The base sequence as shown in SEQ ID NO: 3 is an amino acid sequence encoded by the base sequence as shown in SEQ ID NO: 2.

[0023]

As used herein, the term "a base sequence in which one or more of bases are deleted from, added to and/or substituted for" refers to a base sequence in which 1 to 20, preferably 1 to 15, more preferably 1 to 10 and still further preferably 1 to 5 bases are deleted, added and/or substituted.

As used herein, the term "an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for" refers to an amino acid sequence in which 1 to 20, preferably 1 to 15, more preferably 1 to 10 and still further preferably 1 to 5 amino acids are deleted, added and/or substituted.

[0024]

As used herein, the term "DNA that can hybridize ...under stringent conditions" refers to the base sequence of DNA found by the colony hybridization method, plaque hybridization method or Southern blot hybridization method using DNA as a probe, which includes DNA that can be identified by performing hybridization at 65°C in the presence of 0.7 ~ 1.0 M NaCl using a filter on which colony or plaque-derived DNA or its DNA fragments are immobilized and then washing the filter at 65°C using 0.1 ~ 2 XSSC solution, wherein the composition of 1 XSSC is 150 mM NaCl and 15 mM sodium citrate.

[0025]

Hybridization can be performed by the methods described in Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; hereinafter referred to as "Molecular Cloning 2<sup>nd</sup> Ed.") and the like.

[0026]

The DNA that can hybridize under stringent conditions includes DNA that has a specified level or more of homology with the base sequence of DNA used as a probe. Here, the term "a specified level or more of homology" means not less than 70%, preferably not less than 80%, more preferably not less than 90%, more preferably not less than 93%, particularly preferably not less than 95% and most preferably not less than 97%. As used herein, the DNA having a specified level or more of homology includes both polynucleotide having the aforementioned homology and its complementary stranded polynucleotide.

[0027]

The DNA according to the present invention is DNA involving in the restoration of fertility from cytoplasmic male sterile individual organisms. More specifically, the invention provides  $F_1$  seeds in which fertility is restored by crossbreeding a transgenic plant (Rf line) introduced with DNA according to the present invention using a gene splicing technique with an individual organism of the cytoplasmic male sterility line (cms line). The cms line preferably includes konase cms and ogura cms.

[0028]

(2) Method for acquiring DNA according to the present invention

The method for acquiring DNA is not limited in the present invention. The DNA according to the invention can be isolated by a general breeding method and a general genetic engineering method well known to people skilled in the art based on information on the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 and information on the amino acid sequence described in SEQ ID NO: 3 as disclosed herein.

[0029]

Specifically, genomic DNA containing a cytoplasmic male fertility restorer gene of a suitable plant in which the gene according to the present invention can be expressed, more specifically a Raphanus plant including radish species or related species and more specifically a Raphanus plant including Kosena radish, Ogura radish, Yuanhong radish, their species and related species can be isolated from Brassica plants into which it is transfused by crossbreeding or cell fusion and more specifically from Rf rapeseeds. For example, the gene according to the present invention can be isolated by first isolating DNA markers positioned in the vicinity of the Rf gene, making a genomic map showing the genetic distance between the DNA markers and the Rf gene, and then using a positional cloning method, which is also referred to as chromosome walking, of the Rf region based on the genomic map.

[0030]

This method starts with finding suitable DNA markers on a genomic DNA and then making a genomic map by measuring the distance between the Rf gene and the DNA markers. The DNA markers, which must be distinguished between paternal and maternal genomes, generally have several hundred bp in length. Also, the DNA markers need to be located on the same chromosome as the gene. It is desirable that the markers are nearly the same as the gene in terms of a mode of inheritance because of the close physical location (i.e. the markers are genetically strongly linked).

[0031]

The RFLP method was previously used as a DNA marker isolation method, but the RAPD method and the AFLP (Amplified Fragment Length Polymorphism) method, simple methods using PCR, has recently been used (Nucleic Acids Research, 1995, Vol. 23, No. 21, 4407-4414). The AFLP method is especially effective as a means of obtaining genetically strongly linked markers. Materials used for measuring the genetic distance between a gene and markers usually include an F2 population obtained by self-fertilizing an F1 generation crossbred between a recessive homo individual, which has no Rf1 gene, and a dominant homo individual, which has an Rf1 gene in homo and a BC1 population obtained by crossbreeding between an F1 generation and its parent (i.e. a recessive homo individual, which has no desired gene).

[0032]

The aforementioned recessive homo individuals include cytoplasmic male sterile Raphanus plants such as radish species and related species, more specifically cytoplasmic male sterile Kosena radish and Ogura radish, and Brassica plants, more specifically cms rapeseeds, transfused with cytoplasmic male sterility derived from Kosena radish (Kosena cms) or cytoplasmic male sterility derived from Ogura radish (Ogura cms).

[0033]

The aforementioned dominant homo individuals include Rf line Raphanus plants such as radish species and related species, more specifically cytoplasmic male fertility restored Kosena radish, Ogura radish and Yuanhong radish, and Brassica plants, more specifically cms rapeseeds, transfused by crossbreeding or cell fusion with a cytoplasmic male fertility restorer gene of Raphanus plants such as radish species and related species.

[0034]

It is desirable to analyze 100 individuals or more and more preferably 1000 individuals or more of an F<sub>2</sub> population obtained by self-fertilizing an F<sub>1</sub> generation crossbred between the aforementioned parents and a BC<sub>1</sub> population obtained by crossbreeding between an F<sub>1</sub> generation and a recessive homo individual. The more the number of individuals, the more precise a genomic map and the shorter the physical distance between DNA markers and a desired gene. This is true for an Rf gene. DNA markers of shorter physical distances can be obtained.

[0035]

Materials used for measuring genetic distances between DNA markers and an Rf gene include an F<sub>2</sub> population of several thousands obtained by self-fertilizing an F<sub>1</sub> generation of radish crossbred between cms line Kosenia radish (*Raphanus sativus* cv. Kosenia) and Rf line Yuanhong radish (*Raphanus sativus* cv. Yuanhong) by a method described in N. Koizuka, et al., *Theor. Appl. Genet.*, 100:949-955, 2000. Analyzing these allows isolating DNA markers linked both sides of the Rf gene at a genetic distance of approximately 0.2 cM. As a result, it is possible to make a genomic map showing the genetic distances between markers and the Rf gene as shown in Fig. 1.

[0036]

Subsequent to making a genomic map, the corresponding genomic DNAs need to be cloned to link DNA markers on both sides of a desired gene. Since the physical distance between DNA markers and a desired gene is usually large, the region between the DNA marker and the desired gene must be covered by linking multiple clones having a genomic DNA fragment. The step of linking DNA markers with clones having a genomic DNA fragment is referred to as making a contig. This is true for a RF gene. A contig can be made by linking multiple clones having a genomic DNA fragment for DNA markers that are physically close to the Rf gene in such a way as to cover the Rf gene region.

[0037]

A group of clones having a genomic DNA fragment can be obtained by making a genomic library. Several types of vectors are usually used depending on the length of genomic DNA to be cloned to make a library using a lambda phage vector that can clone a fragment up to approximately 20 kb, a cosmid vector that can clone a relatively long fragment (~ 40 kb), BAC (Bacterial Artificial Chromosome) vector that can clone a longer fragment (100 kb or more) or the like.

[0038]

It is important for any library that a value calculated by multiplying an average length of cloned fragments by a population number of a library should be approximately four to five times more than the total length (a genome size) of a genome provided to the library. The genome size of radish is believed to be approximately 500 Mbp. In the case of a lambda phage vector, if an average length is 20 kb, a population number is in the range of  $1.0 \times 10^5 \sim 1.25 \times 10^5$ . In the case of a cosmid library, if an average length is 40 kb, a population number is in the range of  $5.0 \times 10^4 \sim 6.25 \times 10^4$ . The genome size of rapeseeds is believed to be approximately 1000 Mbp. In the case of a lambda phage vector, if an average length is 20 kb, a population number is in the range of  $2.0 \times 10^5 \sim 2.5 \times 10^5$ . In the case of a cosmid library, if an average length is 40 kb, a population number is in the range of  $1.0 \times 10^5 \sim 1.25 \times 10^5$ .

[0039]

Genomic DNA to be provided to a library can be extracted from an organism containing a specific gene. In the case of a Rf gene, what can be used include Rf line Raphanus plants such as radish species and related species, more specifically cytoplasmic male fertility restored Kosenia radish, Ogura radish and Yuanhong radish, and Brassica plants, more specifically cms rapeseeds, transfused by crossbreeding or cell fusion with a cytoplasmic male fertility restorer gene of Raphanus plants such as radish species and related species. In general, it is most desirable to make a genomic library by extracting genomic DNA from a plant having the same Rf line as the parent used at the time of making an F2 population and a BC1 population. Genomic DNA can be prepared by an ordinary method such as the CTAB method (Murray, M. G. and Thompson, W. F. (1980), Nucleic Acids Res., 8, 4321).

[0040]

A contig is made by isolating clones retaining a DNA marker on both sides of an Rf gene first. The clones are isolated by an ordinary method based on a genomic library. In the case of a lambda phage library, the plaque hybridization method is used. In the case of a cosmid library and a BAC library, the colony hybridization method is used. Next, using the terminal regions of an isolated clone as an indicator, a clone adjacent to the clone is isolated in order to make a contig. After making a contig, the base sequence of the contig is determined by an ordinary method.

[0041]

As genome projects have recently made great progress, so does technology to assume functional genes based on the base sequence of genomic DNA. Gene discovery programs represented by "Genscan" can assume genes with very high accuracy. Also, homology research programs represented by "Blast" can allow assuming the similarity of other genes and proteins. Thus, specific genes have been assumed and isolated using such analysis software. When it comes to an Rf gene, it is thought it possible to isolate genomic DNA and identify its base sequence using similar analysis software. Such an analysis allows clarifying a promoter on the base sequence of genomic DNA, a structural gene including intron and a terminator as well as a gene without intron (i.e. a gene to be translated into protein) and an amino acid sequence encoded by the gene. Thus, an Rf gene on a contig can be assumed with very high accuracy.

[0042]

It is possible to determine whether or not the promoter, structural gene containing intron and terminator thus obtained are the Rf gene itself based on the aforementioned genomic map, the relationship between DNA markers and the relationship between DNA markers and a contig.

The actual expression of a specific genome in a living body can be proved by purifying mRNA and isolating the complementary DNA (i.e. cDNA). The initiation of transcription can be proved by the 5' – RACE method, a simple method using PCR, or a primer extension method and an S1 mapping method as more accurate methods.

The abovementioned methods are described in Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989 and the like.

[0043]

Genes to be translated into protein that can be assumed by the aforementioned method include DNA described in SEQ ID NO: 2. Based on the DNA sequence, cDNA can also be isolated by a general genetic engineering method.

[0044]

Specifically, a cDNA is prepared by an ordinary method from Brassica plants and more specifically from Rf rapeseeds transfused with genomic DNA of a suitable plant in which the gene according to the present invention can be expressed, more specifically a Raphanus plant including radish species or related species and more specifically a Raphanus plant including Kosen radish, Ogura radish, Yuanhong radish, their species and related species. The cDNA corresponding to the gene of the present invention can be isolated by selecting specific clones based on the library using a proper DNA fragment unique to the gene according to the invention as a probe or an antibody of the translated product of the gene according to the invention.

[0045]

The source of cDNA includes various cells, tissues and cultured cells derived from them that express the gene according to the invention. The isolation of total RNA, the isolation and purification of mRNA and the acquisition and cloning of cDNA can be performed by an ordinary method.

A method for screening the gene according to the invention is not limited. Any ordinary method can be used.

[0046]

A probe that can be used here includes DNA chemically synthesized based on information about the base sequence of the gene according to the invention and the gene according to the invention that has already been acquired and its fragments. A sense primer and an antisense primer made based on information about the base sequence of the gene according to the invention can be used as a probe for screening.

[0047]

The nucleotide sequence of a sense primer and an antisense primer used as a probe as described above is a partial nucleotide sequence corresponding to DNA encoding the amino acid sequence expressed by SEQ ID NO: 3 and has at least continuous bases of not less than 15, preferably continuous bases of not less than 20, more preferably continuous bases of not less than 30 and most preferably continuous bases of not less than 50. Or, a positive clone having the aforementioned sequence can also be used as a probe.

[0048]

The gene according to the present invention can be obtained by combining ordinary methods used for the isolation of genes such as a DNA/RNA amplification method based on the PCR method and the RACE method represented by the 5' – RACE method.

[0049]

A primer to be used for PCR can optionally be set and synthesized by an ordinary method based on information about the sequence of the gene according to and revealed by the invention. The isolation and purification of amplified DNA/RNA fragments can follow an ordinary method as described above including gel electrophoresis.

[0050]

Furthermore, the base sequences of the gene according to the invention thus obtained or various DNA fragments can be determined by an ordinary method.

The presence of the gene according to the invention and the presence or absence of expression in individual organisms or various tissues can characteristically be detected using part or whole of the base sequence of the gene, for example.

[0051]

As described above, the gene according to the present invention includes DNA encoding the amino acid sequence expressed in SEQ ID NO: 3, but is not limited to this. Included are homologues of the gene as well.

As used herein, the term "homologues of the gene" refers to a group of related genes recognizable as one gene family based on sequence homology with the gene according to the invention (or its genetic products), the aforementioned structural characteristics and the similarity of biological functions including the allele gene of the gene.

[0052]

The gene according to the present invention is not limited to a gene having a specific base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2. It can be a base sequence formed by optionally combining codons for amino acid residues described in SEQ ID NO: 3. Codons can be selected by an ordinary method in consideration of the frequency of the use of codons by a host, for example.

[0053]

As described above, the gene according to the present invention also includes DNA that can hybridize with DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 or its part under stringent conditions. Such DNA has a specific level or more of homology with DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 or its part.

[0054]

The aforementioned DNA having a specific level or more of homology refers to polynucleotide and its complementary chain polynucleotide having at least 70% identity, preferably at least 90% identity, more preferably at least 95% identity and still further favorably at least 97% identity with the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 or its part or the base sequence encoding the amino acid sequence described in SEQ ID NO: 3 or its part.

[0055]

More specifically, it is exemplified by DNA having a base sequence that hybridizes with DNA having the base sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2 under the stringent conditions of 50°C in 0.2 XSSC containing 0.1% SDS or 60°C in 1 XSSC containing 0.1% SDS.

[0056]

Particularly, among the DNAs according to the present invention, the following DNAs can be made by any method well known to people skilled in the art including chemical synthesis, genetic engineering and mutagenesis:

- a DNA that can hybridize with a DNA having the base sequence described in SEQ ID NO: 1 or its part under stringent conditions and involves in the restoration of fertility from cytoplasmic male sterility;

- a DNA having a base sequence in which one or more of bases are deleted from, added to and/or substituted for the base sequence described in SEQ ID NO: 2 or its part and involving in the restoration of fertility from cytoplasmic male sterility; and

- a DNA encoding a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 or its part and involving in the restoration of fertility from cytoplasmic male sterility. For example, mutated genes can be obtained by carrying out the mutagenesis of DNAs having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2.

[0057]

Mutated genes can be produced by any well-known method such as a method using random mutants, mutants having a target or synthesized genes (New Genetic Engineering handbook, Experimental Medicine, Additional Volume, Yodosha Co., Ltd.).

Included are a method of contacting DNA having the base sequence described in SEQ ID NO: 1 or SEQ ID NO: 2 with a chemical (a mutagen), a method of irradiating ultraviolet and genetically engineering methods. The site-specific mutagenesis, one of genetically engineering methods, is very useful because it is a method of introducing a specific mutation into a specific position and can be carried out in accordance with the method described in Molecular Cloning 2<sup>nd</sup> Ed.

[0058]

(3) A vector containing the DNA according to the present invention

The DNA according to the present invention can be used as a recombinant vector by recombining it into a proper vector. The A vector can be either an expression vector or a non-expression vector and can be selected depending on the purpose.

A cloning vector is preferably one that can autonomously replicate in the Escherichia coli k12 strain including a phage vector and a plasmid vector. An expression vector for Escherichia coli can also be used as a cloning vector. Specifically, the following are included: ZAP Express [manufactured by Stratagene Corporation, Stratagies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP 11 (manufactured by Stratagene Corporation),  $\lambda$ gt10,  $\lambda$ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)],  $\lambda$ TriplEx (manufactured by Clone Tech Corporation),  $\lambda$ Excell (manufactured by Pharmacia Corporation), pT7T318U (manufactured by Pharmacia Corporation), pcD2 [Mol. Gen. Biol., 3, 280 (1983)], pMW218 (manufactured by Wako Pure Chemical Industries, Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pEG400 [J. Bac, 172, 2392 (1990)] and pQE-30 (manufactured by Qiagen Corp.).

[0059]

An expression vector can be selected in consideration of the combination with a host and is preferably one that can autonomously replicate in the host cell or can be recombined into a chromosome and contains a promoter in the position that allows transcribing the gene according to the present invention.

In the case that a bacterium is used as a host cell, an expression vector that allows expressing DNA is preferably a recombinant vector that can autonomously replicate in the bacterium and is composed of a promoter, a ribosome binding sequence, the aforementioned DNA and a transcription termination sequence. A promoter controlling gene may be contained.

[0060]

Expression vectors for bacteria include pBTrP2, pBTac1, and pBTac2 (these three are available from Boehringer Mannheim Corporation), pKK233-2 (manufactured by Pharmacia Corporation), pSE280 (manufactured by Invitrogen Corporation), pGEMEX-1 (manufactured by Promega Corporation), pQE-8 (manufactured by Qiagen Corp.), pQE-30 (manufactured by Qiagen Corp.), pKYP10 (Japanese Unexamined Patent Publication No. S58-110600), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], PLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescriptII SK+, pBluescriptII SK(-) (manufactured by Strategene Corporation), pTrs30 (FERMBP-5407), pTrs32 (FERM BP-5408), pGEX (manufactured by Pharmacia Corporation), pET-3 (manufactured by Novagen Corporation), pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTp5, pC194, pUC18 [Gene, 33, 103 (1985)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (manufactured by Takara Shuzo Co., Ltd.), pSTV29 (manufactured by Takara Shuzo Co., Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pPA1 (Japanese Unexamined Patent Publication No. S63-233798), pEG400 [J. Bacteriol., 172, 2392 (1990)] and pQE-30 (manufactured by Qiagen Corp.). Promoters for bacteria include promoters originated from Escherichia coli and phages such as a trp promoter (P trp), a lac promoter (P lac), a P<sub>L</sub> promoter, a P<sub>R</sub> promoter and P<sub>SE</sub> promoter, a SP01 promoter, a SP02 promoter and a penP promoter.

[0061]

Expression vectors for yeasts include Yep13 (ATCC37115), Yep24 (ATCC37051), Yep50 (ATCC37419), pHS19 and pHS15. Promoters for yeasts include a PH05 promoter, a PGK promoter, a GAP promoter, an ADH promoter, a gal1 promoter, a gal10 promoter, a heat shock protein promoter, an MFa1 promoter and a CUP1 promoter.

[0062]

Expression vectors for animal cells include pcDNA1, pcDM8 (available from Funakoshi Corporation), pAGE107 (Japanese Unexamined Patent Publication No. H3-22979; Cytotechnology, 3, 133, (1990)), pAS3-3 (Japanese Unexamined Patent Publication No. H2-227075), pCDM8 [Nature, 329, 840 (1987)], pc DNAI/AmP (manufactured by Invitrogen Corporation), pREP4 (manufactured by Invitrogen Corporation), pAGE103 [J. Biochem., 101, 1307 (1987)] and pAGE210. Promoters for animal cells include a promoter for an IE (immediate early) gene of cytomegalovirus (human CMV), a promoter for retrovirus, a metallothionein promoter, a heat shock promoter and a SRA promoter.

[0063]

Expression vectors for plant cells include pIG121-Hm [Plant Cell Report, 15, 809-814 (1995)], pBI121 [EMBO J. 6, 3901-3907 (1987)] and pLAN411 and pLAN421 [Plant Cell Reports 10 (1991) 286-290]. When a long DNA fragment of 10 kb or more is introduced into a plant, it is preferred to use a vector improved to allow retaining and introducing long chain DNA stably including pBIBAC2 (Gene 200 (1997) 107-116), pYLTAC7 (PNAS 96 (1999) 6535-6540) and pBGRZ2 (Bioscience and Industry 55 (1997) 37-39). Promoters for plant cells include a cauliflower mosaic virus 35S promoter [Mol. Gen. Genet (1990) 220, 389-392]. The transformation of plants will be described in detail below.

[0064]

(4) A transformant having the DNA according to the present invention

A transformant having the DNA according to the present invention can be made by introducing the aforementioned vector and preferably an expression vector into a host.

Host bacterial cells include genus *Escherichia*, genus *Corynebacterium*, genus *Brevibacterium*, genus *Bacillus*, genus *Microbacterium*, genus *Serratia*, genus *Pseudomonas*, genus *Agrobacterium*, genus *Alicyclobacillus*, genus *Anabaena*, genus *Anacystis*, genus *Arthrobacter*, genus *Azobacter*, genus *Chromatium*, genus *Erwinia*, genus *Methylobacterium*, genus *Phormidium*, genus *Rhodobacter*, genus *Rhodopseudomonas*, genus *Rhodospirillum*, genus *Scenedesmus*, genus *Streptomyces*, genus *Synneococcus* and genus *Zymomonas*. The methods for introducing a recombinant vector into a bacterial host include a method using a calcium ion and a protoplast method.

[0065]

Host yeast cells include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans* and *Schwanniomyces alluvius*.

Any method for introducing a recombinant vector into a host yeast cell can be used including an electroporation method, a spheroplast method and a lithium acetate method.

[0066]

Host animal cells include Namalwa cells, COS1 cells, COS7 cells and CHO cells.

Any method for introducing a recombinant vector into an animal cell can be used including an electroporation method, calcium phosphate method and a lipofection method. Transformants using plant cells will be described below.

[0067]

(5) Production of protein according to the present invention

The present invention relates to any one of the following proteins:

- (1) a protein having the amino acid sequence described in SEQ ID NO: 3 or the amino acid sequence from the 84<sup>th</sup> residue to the 804<sup>th</sup> residue described in SEQ ID NO: 3 ; or
- (2) a protein having an amino acid sequence in which one or more of amino acids are deleted from, added to and/or substituted for the amino acid sequence described in SEQ ID NO: 3 or the amino acid sequence from the 84<sup>th</sup> residue to the 804<sup>th</sup> residue described in SEQ ID NO: 3 and involving in the restoration of fertility from cytoplasmic male sterility.

[0068]

The protein according to the present invention can be acquired by culturing a transformant containing the gene according to the present invention, allowing producing and accumulating the protein according to the present invention in the culture, and then harvesting the protein from the culture.

[0069]

A transformant containing the gene according to the present invention can be cultured by any ordinary method used for culturing a host.

If the transformant according to the present invention is prokaryote such as *Escherichia coli* or eukaryote such as yeast, a medium used for culturing these microorganisms may be a natural or synthetic medium as far as the medium contains carbon sources, nitrogen sources, inorganic salts and the like that can be utilized by the microorganisms and allows efficiently culturing a transformant. The culture condition is preferably aerobic such as shake culture and submerged culture. The culture temperature is usually in the range of 15 ~ 40°C. The culture time is usually 16 hours to 7 days. During the culture, pH should be maintained 3.0 ~ 9.0, which can be adjusted using inorganic or organic acids, alkali solution, urea, calcium carbonate, ammonia or the like. As the need arises, a medium can be added with antibiotics such as ampicillin and tetracycline.

[0070]

A transformant acquired by using an animal cell as a host cell can be cultured in any widely used medium such as a RPM 11640 medium [The Journal of the American Medical Association, 199, 519 (1967)], an Eagle's MEM medium [Science, 122, 501 (1952)], a DMEM medium [Virulorogy, 8, 396 (1959)], a 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] and media made by adding fetal bovine serum to these media. The culture is usually performed at pH 6 ~ 7 at 30 ~ 40 °C for 1 to 7 days in the presence of 5% CO<sub>2</sub>. As the need arises, a medium can be added with antibiotics such as kanamycin and penicillin.

[0071]

A transformant acquired by using a plant cell as a host cell can be cultured in a MS medium, a R2P medium or any other media usually used depending on the type of plant species. The culture is usually performed at pH 6 ~ 8 at 15 ~ 35°C for 1 to 21 days. As the need arises, a medium can be added with antibiotics such as kanamycin and hygromycin.

[0072]

The protein according to the present invention involving in the restoration of fertility from cytoplasmic male sterile individual organisms can be isolated from the culture containing a transformant and then purified using ordinary isolation and purification methods for protein.

For example, if the protein according to the present invention is expressed in cells in the state of dissolution, the cells are collected by centrifugal separation after the completion of the culture, suspended in an aqueous buffer solution, and then smashed with a French press, a Manton Gaulin homogenizer or a Dyno-mill to obtain a cell free extract. Purified samples can be produced from the supernatant obtained by performing the centrifugal separation of the cell free extract using an ordinary protein isolation and purification method such as a solvent extraction method, a salt precipitation method using ammonium sulfate or the like, a desalination method, a precipitation method by an organic solvent, anion exchange chromatography using resin including diethylaminoethyl (DEAE) Sepharose and DIAION HPA-75 (Mitsubishi Kasei Corporation), cation exchange chromatography using resin including S-Sepharose FF (Pharmacia Corporation), hydrophobic chromatography using resin including Butyl Sepharose and Phenyl Sepharose, a gel filtration method using molecular sieves, affinity chromatography, a chromatofocusing method or electrophoresis including isoelectric electrophoresis or a combination thereof.

[0073]

If protein is expressed in cells in the form of insoluble substance, the cells are collected, smashed and then centrifuged. The protein is collected from the precipitated fraction. Then, the insoluble protein is solubilized by a protein denaturing agent. The solubilized solution is dialyzed or diluted into its normal stereostructure with a solution containing no protein denaturing agent or a solution in which the concentration of a protein denaturing agent is so diluted that it cannot denature protein to change the protein. Subsequently, purified samples can be produced by the same isolation and purification method as described above.

[0074]

If the protein according to the present invention or its derivative such as sugar modification is secreted extracellularly, the protein or its derivative such as a glycosylated derivative can be collected from the supernatant of the culture. In other words, the culture is processed by the centrifugal separation method and the like, as described above, to obtain a soluble fraction, and then purified samples can be produced from the soluble fraction by the same isolation and purification method as described above.

[0075]

Furthermore, the protein according to the present invention can be produced by chemical synthesis such as an Fmoc method (a Fuluorenyl methyloxy carbonyl method) and a tBoc method (a t-butylloxy carbonyl method). Also, the protein can be synthesized using peptide synthesizers such as those obtainable from Sowa Trading Co., Inc. (manufactured by Advanced Chem Tech Corporation in USA), Perkin-Elmer Japan (manufactured by Perkin-Elmer Corporation in USA), Pharmacia Biotech (manufactured by Pharmacia Biotech Corporation in Sweden), Aloka (manufactured by Protein Technology Instrument Corporation in USA), Kurabo Industrires Ltd. (manufactured by Synthecell-Vega Corporation), Nihon PerSeptive Ltd. (PerSeptive Corporation in USA) and Shimadzu Corporation.

[0076]

(6) Plant transformants containing the DNA according to the present invention

The base sequence described in SEQ ID NO: 1 is the original plant genomic base sequence. This base sequence operably contains a promoter and a terminator necessary for the expression of a gene. In the case of the direct introductory method, the gene can be cloned into a general cloning vector such as cosmid pWE15 (manufactured by Strategen Corporation). In the case of using *Agrobacterium*, the gene can be cloned into a general vector for plant transformation such as pBI121 (manufactured by Clontec Corporation).

[0077]

Also, plant cells can be introduced with DNA having a base sequence formed by removing some introns from the aforementioned base sequence, DNA having a base sequence formed by removing almost all introns from the aforementioned base sequence, DNA described in SEQ ID NO: 2 or a portion corresponding to its 250<sup>th</sup> ~ 2415<sup>th</sup> base or DNA encoding protein described in SEQ ID NO: 3 or a portion corresponding to its 84<sup>th</sup> residue ~ 804<sup>th</sup> residue.

[0078]

Moreover, the promoter and terminator may be substituted by a promoter and a terminator that function in the well-known plant cells.

In the case that DNA described in SEQ ID NO: 2 or a portion corresponding to its 250<sup>th</sup> ~ 2415<sup>th</sup> base or DNA encoding protein described in SEQ ID NO: 3 or a portion corresponding to its 84<sup>th</sup> residue ~ 804<sup>th</sup> residue is introduced into plant cells, a promoter and a terminator are required in addition to the DNA. A general expression vector used very often includes pBI121 (manufactured by Clontec Corporation). This vector uses a 35S promoter of cauliflower mosaic virus as a promoter and a Nopaline synthase terminator in the Ti plasmid of *A. tumefaciens* as a terminator. A promoter necessary for the expression may be an rbcS promoter that widely exists in plants besides the aforementioned 35S promoter of cauliflower mosaic virus, more preferably a promoter that expresses in the growing period of pollens and still further preferably the original promoter existing in the upstream of the gene. A promoter may be a 35S terminator of cauliflower mosaic virus besides the aforementioned Nopaline synthase terminator and more preferably the original terminator existing in the downstream of the gene.

[0079]

In the following Examples, the present inventors made vectors for plant transformation in order to introduce into a plant the original form of DNA for an Rf gene containing introns between the original promoter and the original terminator in the genome as described in SEQ ID NO: 1. The base sequence described in SEQ ID NO: 1 was cut out of a To3-2 clone, part of the contig, with a restriction enzyme and subcloned to a proper cloning vector. The subcloned fragment was introduced into pKM424, a vector for plant transformation, to obtain a vector that allows introducing the fragment into a plant. This vector was introduced into *Agrobacterium* for plant transformation. Thus, the DNA fragment could be introduced into a plant genome by infecting a plant with the *Agrobacterium* containing this vector.

[0080]

Plants used for the gene according to the present invention include oil crops such as rapeseed, sunflower, soybean and palm, cereals such as rice, corn and wheat, flowering plants such as tobacco and petunia and various vegetables such as tomato, broccoli, cabbage, Chinese cabbage and carrot,

Among those plants, tomato and genus *Brassica* including rapeseed, cabbage, Chinese cabbage and broccoli are preferred. Particularly, rapeseed, cabbage, Chinese cabbage and broccoli are preferred. The most preferred plant is rapeseed.

[0081]

In the present invention, the transgenic plant source includes seeds, sprouts, seedlings, calluses, cultured cells and plant bodies. For example, plant parts to be selected are as follows: sprouts or protoplast in rapeseed; sprouts, calluses or cultured cells in soybean; sprouts in sunflower; calluses or cultured cells in palm; sprouts, calluses, cultured cells or protoplast in rice; sprouts, seedlings, calluses, cultured cells or protoplast in corn; sprouts, calluses or cultured cells in wheat; sprouts, calluses, cultured cells or protoplast in cabbage and broccoli; and sprouts, calluses, cultured cells or protoplast in carrot. Thus, plant parts may optionally be selected depending on the type of plants as usually performed by people skilled in the art.

[0082]

Plants may be transformed by any ordinary method. For example, a vector can be introduced into a plant by first introducing the vector into *Agrobacterium* and then infecting a plant with the *Agrobacterium*. Or, a vector can directly be introduced into cells using an electroporation method, a DEAE dextran method, a calcium phosphate method, a polyethylene glycol method or a particle gun method.

[0083]

A description of a gene introduction method preferred for rapeseed is given below.

The hypocotyls of rapeseed germinated sterilely in the MS medium containing sugar such as sucrose as a carbon source is precultured in the medium containing 2,4 – dichlorophenoxyacetic acid and sucrose. *Agrobacterium* proliferated in the YEB medium is collected by centrifugation and resuspended in the MS medium containing sucrose. The suspension is added with the hypocotyls of rapeseed to be shaken. The hypocotyls thus obtained are brought back to the preculture medium to cocultivate for three days. Subsequently, they are transferred to a selective medium containing plant hormones such as zeatin and benzylaminoprine for selection. The green regeneration buds thus obtained are cultivated in an elongation medium optionally containing plant hormones such as benzylaminoprine followed by a rooting medium optionally containing naphthaleneacetic acid and plant hormones such as benzylaminoprine to obtain regenerated individual organisms. The crossbreeding of these individual organisms with individual organisms of the cms line provides a fertility-restored F1 hybrid.

[0084]

Thus, the introduction of the DNA according to the present invention into plants allows restoring fertility from cytoplasmic male sterile individual organisms.

The confirmation of expression is possible by crossbreeding the aforementioned regenerated individual organisms with rapeseed of the cms line and then examining the fertility of its offspring. It is, however, preferable to perform transformation by using rapeseed containing the cms cytoplasm as raw material in terms of time and operation, because fertility can be examined after transferring the aforementioned transformants (regenerated individual organisms) that have formed roots to soil containing ordinary fertilizer to make them bloom.

[0085]

If the aforementioned transformation is performed using cells or tissues and preferably hypocotyls, seminal leaves, leaves, pollens, cultured cells, calluses or protoplasts of rapeseed containing the cms cytoplasm, sterility-restored plants can be obtained by transferring the plants (regenerated individual organisms) acquired above to soil containing ordinary fertilizer to make them bloom.

That is, plants having a nucleus introduced with DNA according to the present invention can be obtained using cms cells by introducing the DNA to the cms cells by the aforementioned gene introduction method, selecting cells having a nucleus introduced with the DNA using a marker such as an antibiotic (e.g. kanamycin) resistant or herbicide resistant selective marker, and then cultivating them in an elongation medium or a rooting medium as described above. Such plants are restored in fertility.

[0086]

The gene involving in the restoration of cytoplasmic male fertility can be detected by using an oligonucleotide primer of 15-mer ~ 50-mer optionally set from a DNA according to any one of claims 1-4 or a probe of at least 15-mer or more consisting of part or whole of a DNA according to any one of claims 1-4 and confirming that the amount of base sequence amplified by the primer or the amount of base sequence detected by the probe is one gene or more in one genome of a biological sample.

[0087]

Specific confirmation methods include a PCR method and a Southern hybridization method. Of them, PCR is preferred. Here, methods described in Molecular Cloning: A Laboratory manual, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; hereinafter referred to as "Molecular Cloning 2<sup>nd</sup> Ed.") or the like should be applied.

[0088]

In order to confirm that there is one gene or more in one genome in the PCR method, the level of amplification must be about the same if the same DNA copy number is used as a template, as a simplified method. More accurately, it can be confirmed with a quantitative PCR method by using any type of primer, as an internal control, that amplifies a gene known to exist one in one genome and comparing the level of amplification of this known gene and the level of amplification of a base sequence by the same primer in the same amount of a biological sample. In the Southern hybridization method, it should be confirmed that the amount of detected DNA is the same or more by comparing DNA in a fertility-restored plant that is known to have one gene of the DNA in one genome and the same amount of DNA in a specific plant sample.

[0089]

A primer used for the PCR method is 15 ~ 50mer oligonucleotide having a sequence that is the same as or complimentary to the DNA sequence described in SEQ ID NO: 1 or SEQ ID NO: 2.

A probe used for the Southern hybridization method is whole or part of 15mer or more of a double-stranded DNA, or whole or part of at least 15mer or more of a single-stranded DNA or its complimentary strand that has the same sequence as the DNA sequence described in SEQ ID NO: 1 or SEQ ID NO: 2, or, as described above, DNA having a specific level or more of homology with the base sequence of DNA used as a probe. As used herein, the term "a specific level or more of homology" refers to 70% or more, for example, preferably 80% or more, more preferably 90% or more, still further preferably 93% or more, particularly preferably 95% or more and most preferably 97% or more. As used herein, the term "DNA having a specific level of homology" refers to both the aforementioned polynucleotide having homology and polynucleotide of its complimentary strand.

[0090]

The aforementioned gene detection method is used not only for confirming that the DNA is introduced into transformants but also as a means of confirming presence or absence of an Rf gene in individual organisms introduced with an Rf gene by crossbreeding. This method allows confirming presence or absence of an Rf gene even before blooming in the case of introducing an Rf gene into a cytoplasmic male sterile individual organism. In the case of introducing an Rf gene into an individual organism having ordinary cytoplasm, the fertility of an individual organism of the next generation must be confirmed that is produced by crossbreeding a cytoplasmic male sterile individual organism with pollens at the time of blooming. However, the present method allows confirming presence or absence of an Rf gene before it. This type of method is generally referred to as the use of a DNA marker or the DNA marker breeding. In the case of an Rf gene, it can be used as a DNA marker for the Rf gene (an Rf marker). As described above, the Rf marker is important in breeding commercial variety using a recombinant individual organism introduced with the DNA or a plant introduced with an Rf gene by crossbreeding as a mother plant.

[0091]

As described above, it is possible to determine if the introduced DNA can function as an Rf gene or not by confirming the restoration of fertility in transformants. In addition, the following method is also available for this purpose.

As described above, an Rf gene restores the fertility of plants by decreasing the amount of ORF125, protein causing cms, or ORF138 protein accumulated in mitochondria. Accordingly, it is possible to determine if the introduced gene is an Rf1 gene or not by confirming a decline in the amount of ORF125 or ORF138 accumulated in the mitochondria of a transformed individual organism.

[0092]

The decline in the amount of ORF125 or ORF138 protein accumulated in mitochondria can be confirmed by the following two conditions: the signal size of an antibody for protein originated from a mitochondria genome used as an internal control (e.g. an antibody to F1-F0ATPase (hereinafter "ATPA")) described in N. Koizuka, et al. Theor Appl Genet, 100:949-955, 2000 is equal between a cytoplasmic male sterility individual organism and a fertility-restored individual organism or a transformant made by introducing the DNA into a cytoplasmic male sterility individual organism; and the amount of accumulation of ORF 125 or ORF 138 protein in the fertility-restored individual organism or the transformant made by introducing the DNA into a cytoplasmic male sterility individual organism decreases by not less than 50%, preferably by not less than 60% and still further preferably by not less than 80% as compared with the amount of accumulation in the cytoplasmic male sterility individual organism.

[0093]

In the buds of cytoplasmic male sterile radish having ORF125, the amount of accumulated ORF125 dramatically decrease by introducing Rf1, a fertility restorer gene, to the point that it can hardly be detected. In the buds of fertility-restored rapeseed formed by introducing a fertility restorer gene by crossbreeding, it was observed that the amount of accumulated ORF125 protein decreased by 80% or more. It was also observed in the present Examples that the amount of accumulated ORF125 protein decreased by 80% or more in the buds of transformed rapeseed formed by introducing the DNA into a cytoplasmic male sterile individual organism.

In the aforementioned method, the antibodies to ORF125 and ORF138 protein can be obtained by the following ordinary method. That is, animals are immunized with these proteins (as antigens) to obtain antisera, and then the immunoglobulin G antibody can be purified using an affinity column bonded with protein A. Those antigens can be obtained by purifying proteins produced by a cytoplasmic male sterile plant or its cultured cells, which are being expressed, by an ordinary method. Or, the antigen can be obtained by introducing an ORF125 or ORF138 gene into an expression vector, having them expressed in *Escherichia coli* or yeast, and purifying them in the same way as described above. Furthermore, peptide made by chemically synthesizing part or full length of ORF125 or ORF138 can be used as an antigen. The antibody to ATPA can also been made in the same way.

[0094]

Furthermore, the expression of DNA according to the present invention can be controlled specifically and temporarily by introducing part or whole of the gene according to the present invention together with an inducible promoter into cells containing cms cytoplasm and DNA according to the present invention. Thus, a new hybrid seed production system can be established that does not require the male sterility maintaining line (maintainer) necessary for the production of hybrid seeds.

[0095]

Since rapeseed of the cms line is sterile, a maintainer in which neither cms nor Rf is involved is required for proliferating and maintaining the cms line. In other words, plants of three lines (i.e. an Rf line, a cms line and a maintainer) are conventionally required for the production of hybrid seeds. However, since an Rf gene was isolated and identified by the present invention, it has become possible to construct the cms line that can be proliferated and maintained with no maintainer by inducing a promoter with a chemical at the time of the production of hybrids in order to control the expression of the restored gene.

[0096]

Specifically, part or full length of the gene according to the present invention is introduced into an externally inducible promoter (e.g. a promoter sensitive to a chemical) in antisense or sense direction, and then cells containing the cms cytoplasm and DNA according to the present invention are transformed using the above-said vector.

Cells containing the cms cytoplasm and DNA according to the present invention can be produced not only by transforming cells containing the cytoplasm with DNA according to the present invention as described above, but also by crossbreeding the cms line and the Rf line.

The aforementioned inducible promoter is known in Japanese Unexamined Patent Publication H6-46697, for example. Also, the aforementioned methods may be used for the preparation of a vector and transformation.

[0097]

The aforementioned transformant containing the cms cytoplasm and DNA according to the present invention and introduced with part or whole of DNA according to the present invention together with an inducible promoter is fertile by virtue of an Rf gene because the promoter has not been induced yet. This line can be maintained by self-fertilization. However, this plant can be used as the cms line at the time of the production of hybrid seeds by applying a chemical that has the ability of inducing the promoter because the expression of the Rf gene is inhibited by the induction of the promoter, whereby the plant become male sterile.

Accordingly, the proliferation and maintenance of the cms line has become possible by self-fertilization using the present method. Of three lines conventionally required for the production of hybrid seeds, the maintainer has become unnecessary, resulting in a significant decrease in the production cost.

A description of examples is given below in further detail. However, the present invention is not limited to those examples.

[0098]

Example 1: Isolation of DNA markers linked to the cytoplasmic male fertility restorer gene and preparation of a genomic map

In order to isolate a fertility restorer gene (an Rf gene), DNA markers in the neighborhood of the Rf gene needs to be isolated and then a genomic map showing the genetic distance between the DNA markers and the Rf gene need to be prepared. To start with, the positional cloning of the Rf region was performed.

[0099]

In order to isolate DNA markers, we performed the AFLP (Amplified Fragment Length Polymorphism) method (Nucleic Acids Research, 1995, Vol. 23, No. 21 4407-4414) and used an AFLP Analysis System I AFLP Starter Primer Kit that complies with the AFLP method. As for the material used for measuring the genetic distance of the markers, we used approximately 2100 individual organisms of an F2 population obtained by self-fertilizing 8 individual organisms of the F1 generation of radish obtained by crossbreeding one individual organism of Kosena radish (*Raphanus sativus* cv. Rosena) of the cms line and one individual organism of Yuanhong radish (*Raphanus sativus* cv. Yuanhong) of the Rf line in accordance with the method described in N. Koizuka, et al. Theor Appl Genet, 100:949 – 955, 2000. As a result, we could isolate five markers on both sides of the Rf gene with a genetic distance of approximately 0.2 cM. Fig. 1 shows the genetic distance between each DNA marker and the Rf gene.

[0100]

Example 2: Preparation of a contig based on the genomic map and an analysis of the Rf gene

Subsequent to the preparation of the genomic map, the genomic DNA corresponding to the position needs to be cloned and DNA markers on both sides of the Rf gene linked. We prepared a contig covering the area between the DNA markers and the Rf gene by linking multiple clones having a genomic DNA fragment since the distance between the DAN markers and the Rf gene was large.

[0101]

A group of clones having a genomic DNA fragment is called a genomic library. We made two types of libraries. We prepared genomic DNA by the CTAB method (Murray, M. G. and Thompson, W. F. (1980) *Nucleic Acids Res.*, 8, 4321) from Yuanhong radish, as a DNA donor, that is the same as the parent used at the time of the preparation of the F2 population. We made a lambda phage library of 20 kb in average length and  $1.5 \times 10^5$  in population number using a  $\lambda$  DASHII vector (manufactured by Strategene corporation) as a lambda vector. We also prepared a cosmid library of 40 kb in average length and  $5.5 \times 10^4$  in population number using a pWEB::TNC vector (manufactured by Epicentre Technologies Corporation) as a cosmid vector.

[0102]

In order to make a contig, we first isolated a lambda clone from the aforementioned lambda phage library by the plaque hybridization method using DNA markers on both sides of the Rf gene. We also isolated a cosmid clone from the cosmid library by the colony hybridization method in order to complete a contig that covers DNA markers on both sides as shown in Fig. 1. Base sequences were determined for cosmid clones NIT7/2 and T03-2, part of the contig, by an ordinary method.

[0103]

Next, we analyzed the base sequences of the cosmid clones NIT7/2 and T03-2, part of the cosmid, in consideration of a parameter for Arabidopsis, which is similar to radish in the DNA sequence and whose genomic sequence was recently determined, using "Genscan" (manufactured by Mitsubishi Space Software Corporation). As a result, we found a promoter portion that was thought to express the Rf gene, a structural gene portion containing introns and a terminator portion. Furthermore, we obtained a gene without introns to be translated into protein and the amino acid sequence of the gene. The promoter portion, structural gene portion containing introns and terminator portion are believed to be the Rf gene itself in consideration of their locations based on the aforementioned genomic map, relationship between DNA markers and the relationship between DNA markers and the contig.

[0104]

Example 3: Subcloning of the Genomic DNA area

We then separated a HpaI-SwaI fragment (8553 bp) of the 1<sup>st</sup> base ~ the 8553<sup>rd</sup> base described in SEQ ID NO: 1 that fully contains the portion from promoter to terminator assumed by "Genscan" using gel electrophoresis with agarose (manufactured by FMC Corporation) used for fragment recovery. We recovered DNA after digesting gel containing the DNA fragment with a gel catabolic enzyme (manufactured by Epicentre Technologies Corporation). We cut the fragment thus obtained with a restriction enzyme BamHI to make a cloning fragment. Then, we subcloned these two types of DNA fragments to pGEM-T easy vectors (manufactured by Promega Corporation) to obtain cds6/pGEM-Teasy and cds6BT/pGEM-Teasy. The following explains this procedure in detail.

[0105]

We added 1µg of NIT7/2 cosmid DNA and 10 unit of a restriction enzyme HpaI (manufactured by Takara Shuzo Co., Ltd.) to 100µl of 1 x K restriction enzyme buffer solution (20 mM Tris-HCl (pH 8.5), 10 mM of MgCl<sub>2</sub>, 1 mM of Dithiothreitol and 100 mM of KCl) and heated the solution at 37°C for one hour.

[0106]

After heating, we added 10 µl of 3 M sodium acetate (pH 5.6) and 250 µl of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 rpm at 4°C for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier and then added 89 µl of sterile water to the recovered DNA precipitate in order to dissolve it.

[0107]

To the dissolved DNA solution, we added 10 µl of 10 X H restriction enzyme buffer solution (500 mM Tris-HCl (pH 7.5), 100 mM MgCl<sub>2</sub>, 10 mM Dithiothreitol and 1000 mM NaCl) and 1 µl of 10 unit/µl restriction enzyme SwaI (manufactured by Takara Shuzo Co., Ltd.) and heated the solution at 25°C for one hour. We then added 11 µl of 10 X loading buffer solution (1% SDD, 50% Glycerol and 0.05% Bromophenol Blue).

[0108]

We mixed 1.2 g of SeaPlaqueGTG agarose (manufactured by FMC Corporation), a low melting point agarose, and 150 ml of 1 X TAE buffer solution (40 mM Tris-acetate and 1 mM EDTA), heated at 100°C to dissolve the agarose, and then cooled to 45°C while stirring. We set combs of 300 mm wide and 1 mm thick in a gel tray of 14 x 15 cm, poured the cooled gel and solidified it. We then poured the DNA added with loading dye into the gel combs and performed electrophoresis at 1 X TAE and 30 V/30 cm for 18 hours.

[0109]

After the electrophoresis, the gel was transferred to 0.5 µg/ml of ethidium bromide / 1 x TAE solution and dyed for 30 minutes. We put the gel on a transilluminator irradiated with long wave UV of 365 nm and cut out a specific fragment of 4126 bp with a sterile scalpel. We then cut the gel into fragments of approximately 1 by 1 millimeters square, transferred them to a 2 ml microtube weighed in advance and weighed the gel.

[0110]

We added 1 µl of 50 X GELase buffer (2M Bis-Tris (pH 6.0) and 2M NaCl) relative to 50 mg of the gel. We then put a tube containing the gel in a dry heat block heated at 68°C and heated it for 10 minutes while putting the tube upside down from time to time and stirring to completely dissolve the gel. We transferred the tube to a dry heat block heated at 45°C and heated for 5 minutes while putting the tube upside down from time and stirring. To this tube, we added 1 unit of GELase (manufactured by Epicentre Technologies Corporation) relative to 200 mg of the gel and heated for 30 minutes in the dry heat block heated at 45°C while putting the tube upside down from time and stirring.

[0111]

We added 10 M ammonium acetate (pH 7.0), which was 1/3 by volume of the gel, stirred and centrifuged at 15000 rpm for 5 minutes. We then transferred the supernatant to a new 2 ml microtube and added ethanol, which was twice by volume of the supernatant. After stirring the tube, we centrifuged at 15000 rpm at 4°C for 20 minutes. We removed the supernatant, added 1 ml of 70% ethanol slowly and centrifuged at 15000 rpm at 4°C for 5 minutes. We then removed the supernatant and dried the precipitate for 5 minutes using a spin vacuum dryer. Subsequently, we added 20 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) to dissolve it completely. This is referred to as a cloning fragment 1.

[0112]

Furthermore, we made a fragment by digesting the cloning fragment 1 with BamHI by the following method.

To 20  $\mu$ l of the DNA solution obtained by the aforementioned method, we added 10  $\mu$ l of 10 X K restriction enzyme buffer solution (200 mM Tris-HCl (pH 8.5), 100 mM of  $MgCl_2$ , 10 mM of Dithithreitol and 1000 mM of KCl), 68  $\mu$ l of  $dH_2O$  and 2  $\mu$ l of 10 unit/ $\mu$ l restriction enzyme BamHI (manufactured by Takara Shuzo Co., Ltd.) and heated at 30°C for one hour. After heating, we added 10  $\mu$ l of 3 M sodium acetate (pH 5.6) and 250  $\mu$ l of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 rpm at 4°C for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier and then added 20  $\mu$ l of sterile water to the recovered DNA precipitate in order to dissolve it. We then added 55  $\mu$ l of sterile water, 10  $\mu$ l of 10 X PCR buffer solution (100 mM Tris-HCl (pH 8.3) and 500 mM KCl), 6  $\mu$ l of 25 mM  $MgCl_2$ , 8  $\mu$ l of 2.5 mM dNTP mix and 1  $\mu$ l of 5 unit/ $\mu$ l rTaq DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), mixed them and heated at 72°C for 30 minutes to add dATP to the 3' terminus.

[0113]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100  $\mu$ l of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we added 20  $\mu$ l of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit. This is referred to as a cloning fragment 2.

[0114]

We mixed 5  $\mu$ l of the purified cloning fragments 1 and 2 obtained above with 1  $\mu$ l of 50 ng/ $\mu$ l pGEM-T easy vector (manufactured by Promega Corporation) and 6  $\mu$ l of DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) I solution and incubated at 16°C for 30 minutes.

[0115]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) together with 100  $\mu$ l of sterile water and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100  $\mu$ l of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0116]

We allowed the recovered DNA in the tube to stand on the ice for cooling, put 30  $\mu$ l of *Escherichia coli* for electroporation (manufactured by Gibco BRL corporation) in the tube and mixed softly. We transferred the *Escherichia coli* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.25 kv, 129  $\Omega$  and 50  $\mu$ F using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500  $\mu$ l of SOC medium (manufactured by Gibco BRL) warmed at 37°C to the cuvette. We transferred the *Escherichia coli* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Escherichia coli* on an LB agar medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 100  $\mu$ g/ml of Ampicillin (manufactured by Wako Pure Chemical Industries, Ltd.), 20  $\mu$ g/ml of X-Gal (Takara Shuzo Co., Ltd.) and 1 mM IPTG (Takara Shuzo Co., Ltd.) and cultured at 37°C for 18 hours or more.

[0117]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 100  $\mu$ g/ml of Ampicillin at 37°C for 18 hours or more. We extracted plasmid DNA from the cultured *Escherichia coli* by an ordinary method. We then confirmed that the specific fragment was cloned to the plasmid DNA by cutting it with a restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) and referred to one originated from the cloning fragment 1 as cds6/pGEM-T easy and one originated from the cloning 2 as cds6BT/pGEM-T easy.

[0118]

We cultured the *Escherichia coli* DH10B containing cds6/pGEM-T easy and cds6BT/pGEM-T easy obtained above in 100 ml of LB medium added with 100  $\mu$ g/ml of Ampicillin at 37°C for 18 hours and then purified by the Alkali SDS method using a Qiagen Midi kit (manufactured by Qiagen Corporation).

[0119]

**Example 4-1: Preparation of a vector for plant transformation (1)**

After cutting *cds6/pGEM-T* easy and *cds6BT/pGEM-T* easy each with a restriction enzyme *EcoRI*, we separated the DNA from the vector by gel electrophoresis using agarose for fragment recovery, cloned the recovered DNA to the *EcoRI* site of a vector *pKM424* for plant transformation (a vector formed by adding a fragment of *CaMV35S* promoter: GUS gene: NOS terminator to *pKM424* is a *pLAN421* vector (Plant Cell Reports 10 (1991) 286-290)) and referred to the vectors for plant transformation as *cds6/pKM424* and *cds6BT/pKM424*, respectively. The following will explain it in detail.

Since *cds6/pKM424* and *cds6BT/pKM424* can be obtained by the same process, we will explain only for *cds6/pKM424* below for convenience sake.

[0120]

We added 1 $\mu$ g of *cds6/pGEM-T* easy DNA and 10 unit of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo Co., Ltd.) to 100  $\mu$ l of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol and 100 mM of NaCl) and heated the solution at 37°C for one hour.

We isolated and recovered an *EcoRI* fragment containing *cds6* from *cds6/pGEM-T* easy in the same way as described in recovering the aforementioned *HpaI-SwaI*.

[0121]

We added 1 $\mu$ g of *pKM424*, a vector for plant transformation, and 10 unit of a restriction enzyme *EcoRI* (manufactured by Takara Shuzo Co., Ltd.) to 100  $\mu$ l of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol and 100 mM of NaCl) and heated the solution at 37°C for one hour. After heating, we added 1  $\mu$ l of 1 M Tris-HCl (pH 8.0) and 1 unit of Bacterial Alkaline Phosphatase (manufactured by Takara Shuzo Co., Ltd.), mixed them and heated at 50°C for one hour for dephosphorylation.

[0122]

We then added phenol/chloroform saturated with 200  $\mu$ l of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and stirred violently. After centrifuging at 15000 rpm for 5 minutes, we transferred the supernatant to a new tube. We repeated the same process to remove protein. We added 20  $\mu$ l of 3 M sodium acetate (pH 5.6) and 500  $\mu$ l of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 at 4°C rpm for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier. We then dissolved the precipitate completely by adding 100  $\mu$ l of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) to make a concentration of 10 ng/ $\mu$ l.

[0123]

We mixed 10 µl of the purified EcoRI fragment, 1 µl of the dephosphorylated pKM424 vector and 11 µl of DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) I solution and incubated at 16°C for 30 minutes.

[0124]

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) together with 100 µl of sterile water and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100 µl of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0125]

We allowed the recovered DNA in the tube to stand on the ice for cooling, put 30 µl of *Escherichia coli* for electroporation (manufactured by Gibco BRL corporation) in the tube and mixed softly. We transferred the *Escherichia coli* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.25 kv, 129 Ω and 50 µF using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500 µl of SOC medium (manufactured by Gibco BRL) warmed at 37°C to the cuvette. We transferred the *Escherichia coli* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Escherichia coli* on an LB agar medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 50 µg/ml of Spectinomycin (manufactured by Sigma Corporation) and cultured at 37°C for 18 hours or more.

[0126]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 50 µg/ml of Spectinomycin at 37°C for 18 hours or more. We extracted plasmid DNA from the cultured *Escherichia coli* by an ordinary method. We then confirmed that the region from BamHI site to HpaI site was cloned to the plasmid DNA by cutting it with a restriction enzyme BamHI (manufactured by Takara Shuzo Co., Ltd.) and referred to it as cds6/pKM424.

We cultured the *Escherichia coli* DH10B containing cds6/ pKM424 in 250 ml of LB medium added with 50 µg/ml of Spectinomycin at 37°C for 18 hours and then purified by the Alkali SDS method using a Qiagen Midi kit (manufactured by Qiagen Corporation).

[0127]

**Example 4-2: Preparation of a vector for plant transformation (2)**

After cutting a lambda clone CHI (See Fig. 2; the length of a cloning fragment is approximately 17 kb) fully containing the base sequence of SEQ ID NO: 1 with a restriction enzyme NotI (manufactured by Takara Shuzo Co., Ltd.) found at a multiple cloning site, we separated the DNA fragment from the vector with electrophoresis using agarose for fragment recovery. We then cloned the recovered DNA fragment to the NotI site of a vector pBGRZ2 (Bioscience and Industry 55 (1997) 37-39) for plant transformation and referred to it as CHI/pBGRZ2, a vector for plant transformation. The following will explain it in detail.

[0128]

We added 1µg of lambda clone CHI DNA and 10 unit of a restriction enzyme NotI (manufactured by Takara Shuzo Co., Ltd.) to 100 µl of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol, 100 mM of NaCl, 0.01% BSA and 0.01% TritonX-100) and heated the solution at 37°C for one hour. We isolated and recovered a NotI fragment of the lambda clone CHI in the same way as described in recovering the aforementioned HpaI-SwaI.

[0129]

We added 1µg of pBGRZ2, a vector for plant transformation, and 10 unit of a restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) to 100 µl of 1 x H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol and 100 mM of NaCl, 0.01% BSA and 0.01% TritonX-100) and heated the solution at 37°C for one hour. After heating, we added 1 µl of 1 M Tris-HCl (pH 8.0) and 1 unit of Bacterial Alkaline Phosphatase (manufactured by Takara Shuzo Co., Ltd.), mixed them and heated at 50°C for one hour for dephosphorylation.

[0130]

We then added phenol/chloroform saturated with 200 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and stirred violently. After centrifuging at 15000 rpm for 5 minutes, we transferred the supernatant to a new tube. We repeated the same process to remove protein. We added 20 µl of 3 M sodium acetate (pH 5.6) and 500 µl of ethanol and stirred the solution. After cooling the solution at -80°C for 5 minutes, we centrifuged at 15000 rpm at 4°C for 15 minutes. We then removed the supernatant, slowly added 1 ml of 70% ethanol, and centrifuged at 15000 rpm at 4°C for 15 minutes. After removing the supernatant, we dried the precipitate for 5 minutes using a spin vacuum drier. We then dissolved the precipitate completely by adding 100 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) to make a concentration of 10 ng/µl.

[0131]

We mixed 10 µl of the purified NotI fragment, 1 µl of the dephosphorylated pBIGRZ2 vector and 11 µl of DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) I solution and incubated at 16°C for 30 minutes.

We transferred the aforementioned reaction liquid to an ultrafilter unit: Microcon-50 (manufactured by Millipore Corporation) together with 100 µl of sterile water and centrifuged at 5000 rpm at 4°C for 20 minutes. We discarded water in the trap, added 100 µl of sterile water, and centrifuged at 5000 rpm at 4°C for 20 minutes again. Then, we removed the filter unit, reversed the direction to attach it to a new microtube, centrifuged at 3000 rpm at 4°C for 5 minutes and recovered DNA from the filter unit.

[0132]

We allowed the recovered DNA in the tube to stand on the ice for cooling, put 30 µl of *Escherichia coli* for electroporation (manufactured by Gibco BRL corporation) in the tube and mixed softly. We transferred the *Escherichia coli* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.25 kv, 129 Ω and 50 µF using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500 µl of SOC medium (manufactured by Gibco BRL) warmed at 37°C to the cuvette. We transferred the *Escherichia coli* to a 10 ml culture tube and cultured at 37°C for one hour by shaking. We then spread the cultured *Escherichia coli* on an LB agar medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 25 µg/ml of Kanamycin (manufactured by Wako Pure Chemical Co., Ltd.) and cultured at 37°C for 18 hours or more.

[0133]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with 25 µg/ml of Kanamycin at 37°C for 18 hours or more. We extracted plasmid DNA from the cultured *Escherichia coli* by an ordinary method. We then confirmed that the specific fragment was cloned to the plasmid DNA by cutting it with a restriction enzyme BamHI (manufactured by Takara Shuzo Co., Ltd.) and referred to it as CHI/pBIGRZ2.

We cultured the *Escherichia coli* DH10B containing CHI/pBIGRZ2 in 250 ml of LB medium added with 25 µg/ml of Kanamycin at 37°C for 18 hours and then purified by the Alkali SDS method using a Qiagen Midi kit (manufactured by Qiagen Corporation).

[0134]

Example 5: Introduction of a vector for plant transformation into *Agrobacterium*

We prepared competent cells of *Agrobacterium* and introduced each of the *cds6/pKM424* vector, *cds6BT/pKM424* vector and *CHI/pBGRZ2* vector obtained in Examples 4-1 and 4-2 into the *Agrobacterium* EHA101 for plant transformation thus prepared. The following will explain this process in detail.

[0135]

We prepared the competent cells for electroporation of *Agrobacterium* EHA101 by the method as shown below. We streaked *Agrobacterium* EHA101 on the LB agar medium and cultured at 28°C for 24 hours or more to obtain single colonies. We put colonies of approximately 1 mm in diameter in a 50 ml centrifuge tube containing 20 ml of the LB medium and cultured at 28°C for 40 hours by shaking. After 40 hours, we opened and then shut the lid of the centrifuge tube and cultured for 4 more hours in the same way. To the tube disposed of the supernatant, we put 40 ml of iced 10% sterile glycerol to resuspend the bacterial cells, centrifuged at 1500 x g at 4°C and harvested the cells. We repeated the same process twice. To the bacterial cells thus obtained, we added 500 µl of iced 10% sterile glycerol to resuspend the cells. Then, we poured 100 µl each of the cells into sterile microtubes. After freezing them with liquid nitrogen, the cells were preserved in a freezer at -80°C.

[0136]

We melted the competent cells for electroporation of *Agrobacterium* EHA101 on the ice, put 40 µl of the electrocompetent cells in a 1.5 ml tube iced in advance, added 100 ng of the plasmid DNA of *cds6/pKM424* or *cds6BT/pKM424* and mixed them mildly. Likewise, we put 40 µl of the electrocompetent cells in a 1.5 ml tube iced in advance, added 100 ng of the plasmid DNA of *CHI/pBGRZ2* and mixed them mildly.

[0137]

We transferred the *Agrobacterium* mixed with the DNA to a cuvette (manufactured by USA Scientific Plastics Corporation) for electroporation (distance between electrodes: 1 mm) cooled on the ice in advance. We performed electroporation under the conditions of 1.44 kv, 129  $\Omega$  and 50  $\mu$ F using Electro Cell Manipulator 600 (manufactured by BTX Corporation), and, immediately afterward, added 500  $\mu$ l of SOC medium (manufactured by Gibco BRL) warmed at 30°C to the cuvette. We transferred the *Agrobacterium* to a 10 ml culture tube and cultured at 37°C for one hour by shaking.

We then spread the cultured *Agrobacterium* introduced with *cds6/pKM424* or *cds6BT/pKM424* on an 2 X LB agar medium (2% Bacto-Tryptone, 1% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 50  $\mu$ g/ml of Kanamycin (manufactured by Wako Pure Chemical Industries, Ltd.), 25  $\mu$ g/ml of Chloramphenicol (manufactured by Wako Pure Chemical Industries, Ltd.), 50  $\mu$ g/ml of Spectinomycin (manufactured by Sigma Corporation) and 2.5  $\mu$ g/ml of Tetracycline (manufactured by Sigma Corporation) and cultured at 28°C for 24 hours or more.

We also spread the cultured *Agrobacterium* introduced with *CHI/pBIGRZ2* on an 2 X LB agar medium (2% Bacto-Tryptone, 1% Bacto-Yeast Extract, 1% NaCl and 1.5% Bacto-Agar) added with 50  $\mu$ g/ml of Kanamycin (manufactured by Wako Pure Chemical Industries, Ltd.), 25  $\mu$ g/ml of Chloramphenicol (manufactured by Wako Pure Chemical Industries, Ltd.) and 30  $\mu$ g/ml of Hygromycin (manufactured by Sigma Corporation) and cultured at 28°C for 24 hours or more.

[0138]

We then cultured white colonies that had appeared on the agar medium in 2 ml of LB medium added with the aforementioned antibiotics suitable for each vector at 30°C for 24 hours or more. We extracted plasmid DNA from the cultured *Agrobacterium* by an ordinary method. We then confirmed that the *cds6BT/pKM424* vector or *CHI/pBIGRZ2* vector was introduced into *Agrobacterium* by cutting it with a restriction enzyme *HindIII* (manufactured by Takara Shuzo Co., Ltd.). The confirmed clones were added with the equal amount of 80% sterile glycerol in the solution cultured for 24 hours, preserved at -80°C and used for the transformation of rapeseed.

[0139]

#### Example 6: Preparation of rapeseed transformants

A description of the transformation of rapeseed is given below. We sterilely processed seeds of CMS rapeseed (SW18) having *orf125*, a gene causing cms originated from radish, with 10% hypochlorite solution and had them germinated on a MS medium containing no hormone (T. Murashige and F. Skoog, *Physical. Plant.* 15:485, 1962). We cut only hypocotyls out of embryo plants of 7 to 14 days old after germination and cut them into small pieces of 3 to 5 mm in length and precultured on the MS medium containing hormones (M5519, Sigma Corporation) + sucrose 3% + 2,4-D 1mg/l and agarose (typel, Sigma Corporation) 4% 23 times for 12 to 16 hours. At the time, we cocultivated with BY-2, a cell line originated from tobacco, for nurse culture.

[0140]

We cultured *Agrobacterium* containing CHI/pBIGRZ2 at 28°C for 8 to 48 hours and have it proliferated up to approximately  $OD_{600} = 1.0$ . We suspended cells of *Agrobacterium* in an MS hormone free liquid medium. We then mixed this *Agrobacterium* solution with the chopped hypocotyls and cocultivated for approximately 20 minutes. After the cocultivation, the hypocotyls from which *Agrobacterium* was removed with filter paper was cultured on an MS basic medium + B5 vitamin (M0404, Sigma Corporation) + sucrose 3% + 2,4-D 1mg/l, for example, for two days for infection. After the infection, we transplanted the hypocotyls to a sterile medium made by adding a 500 mg/l concentration of carbenicillin, an antibiotic (geopen, Phizer or Japan, Inc. or carbenicillin disodium salts, GIBCO-BRL Corporation) to an MS basic medium + B5 vitamin + sucrose 3% + 2,4-D 1mg/l in order to remove *Agrobacterium*.

[0141]

Five days to one week after the culture on the aforementioned sterile medium, we cultured the hypocotyls on a MS basic medium + B5 vitamin + sucrose 1% + benzylamino purine 3 mg/l + carbenicillin 500 mg/l added with 5 mg/l of silver nitrate or 5-30 mg/l of kanamycin (kanamycin sulfate, Nacalai Tesque, Inc.) for selection for 14 to 21 days. Whenever green calluses appear, they were transplanted to a medium on the following step.

[0142]

A medium on the following step may be a medium for selection containing an MS medium (M5519, Sigma Corporation) + sucrose 1% + benzylamino purine 3 mg/l + Zeatin 1 mg/l + carbenicillin 500 mg/l + kanamycin 5 ~ 30 mg/l. We transplanted the hypocotyls forming a callus on the cut area to this medium and cultured at 23°C for three weeks. Subsequently, we repeated transplantation 3 to 5 times every three weeks until a green callus appeared.

[0143]

Whenever we found a green callus, we cut it out of its hypocotyl and transplanted it to a medium of the same composition. Subsequently, indefinite buds were formed with 1 to 30% of probability when we cut out only green portions and transplanted them continually. We cultivated the indefinite buds on a B5 basic medium (G5893, Sigma Corporation) + sucrose 3% + benzylamino purine 1 mg/l and, subsequently, had them take roots on a medium containing an MS medium (M5519, Sigma Corporation) + sucrose 3% + naphthalenic acid 0.1 mg/l + benzylamino purine 0.01 mg/l.

[0144]

Example 7: Analysis of transformants (detection of introduced DNA)

We took one leaf from a transformant obtained in Example 6 that had a bud and isolated DNA using a DNA isolation kit (DNeasy plant mini) manufactured by Qiagen Corporation.

We detected three portions of an introduced DNA fragment (sites a, b and c) using a PCR method (Fig. 3 shows the results). The site a has 568 bp from 3186 bp to 3753 bp of the base sequence of SEQ ID NO: 1. We used "5' - GAAGCAAAAAAGAAAACGAGCAGAG - 3'" (SEQ ID NO: 4) as a forward primer and "5' - CCAAAAATCCGAATCCGAATAGAC - 3'" (SEQ ID NO: 5) as a reverse primer. The site b has 244 bp from 4869 bp to 5112 bp of the base sequence of SEQ ID NO: 1. We used "5' - CTCGGCTCTGGGTTTAGTGA - 3'" (SEQ ID NO: 6) as a forward primer and "5' - TCCACAAACCCTAGCCAACA - 3'" (SEQ ID NO: 7) as a reverse primer. The site c has 485 bp from 7766 bp to 8250 bp of the base sequence of SEQ ID NO: 1. We used "5' - GCTTATGCTTCTCTGGTTCGCCTC - 3'" (SEQ ID NO: 8) as a forward primer and "5' - CTCAGTTTTCGTCACCTTACACAATGC - 3'" (SEQ ID NO: 9) as a reverse primer.

[0145]

To 1 µl of transformant DNA solution (50 ng/µl), we added 12. 1 µl of sterile water, 2 µl of 10 x PCR buffer solution (100mM Tris-HCl (ph 8.3) and 500 mM KCl), 1.2 µl of 25 mM MgCl<sub>2</sub>, 1.6 µl of 2.5 mM dNTP mix, 1 µl of 10 µM forward primer solution of each site, 1 µl of 10 µM reverse primer solution of each site, 0.1 µl of 5 unit/µl rTaq DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), mixed them and amplified DNA by repeating 35 cycles at 94°C for 40 seconds, 55°C for 30 seconds and 72°C for one minute. We used UNOII (manufactured by Biometra Corporation) as a thermal cycler. After the reaction, we confirmed the amplified products by gel electrophoresis using 4% Nusive 3:1 Agarose (manufactured by FMC Corporation) / 1 X TBE (89 mM Tris-borate, 89 mM boric acid and 2mM EDTA) buffer solution.

[0146]

As a result, we found that the site a had not been introduced into this transformed rapeseed. With regard to the remaining two sites (i.e. sites b and c), we obtained amplified products that are the same in size as the positive control. Thus, we confirmed that the DNA had been introduced into the transformed rapeseed.

[0147]

Example 8: Analysis of transformants (A decrease in the accumulated amount of ORF125, protein causing cms, confirmed)

We picked one bud from the same plant as in Example 7 and analyzed a decrease in the accumulated amount of ORF125, protein causing cms, by the Western blotting method. Fig. 4 shows the results.

[0148]

(1) Extraction of protein from transformed individual organisms

As far as the extraction of protein and the Western blotting method are concerned, we followed the method of N. Koizuka (Theor Appl Genet (2000) 100:949-955).

Specifically, we put one bud of the transformed rapeseed of 1 mm in length and 100 µl of iced protein extraction buffer solution (50 mM Tris-HCl (pH 7.5) and 2% (W/V) SDS) in an iced mortar and smashed it with a pestle. We then transferred the solution to a microcentrifuge tube and centrifuged at 15000 rpm at 4°C for 15 minutes. After the centrifugation, we transferred the supernatant to a new microcentrifuge tube and heated at 100°C for 5 minutes. We centrifuged again at 15000 rpm at 4°C for 15 minutes and transferred the supernatant to a new microcentrifuge tube to make a SDS soluble protein solution. We then measured the concentration of the SDS soluble protein solution using a protein quantitative kit by the Bradford method. We also measured the concentrations of SDS soluble protein solution extracted from the buds of rapeseed of the cytoplasmic male sterile line and the fertility-restored line.

[0149]

(2) Isolation of protein by the SDS-PAGE method and transfer to PVDF membrane: Western blotting

We separated 15 µg per lane of SDS soluble protein by electrophoresis using 10% SDS polyacrylamide gel of 7 x 10 centimeters square. We also separated a diluted line for rapeseed of the cytoplasmic male sterile line. The condition of electrophoresis was at 10 mA for one hour and 15 mA for one hour. After the electrophoresis, we transferred the protein in the polyacrylamide gel to PVDF membrane (manufactured by Millipore Corporation) at 100 mA for one hour.

[0150]

(3) Detection of protein using an antibody: Western blotting

We divided the PVDF membrane transferred with protein into two upper and lower sheets, transferred them to 10 ml of blocking solution (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Tween 20 and 5% skim milk) and shook the solution for one hour for blocking.

We detected ATPA on the upper PVDF membrane as the control for the weight of mitochondria protein and ORF125, cytoplasmic male sterility-related protein, on the lower PVDF membrane. We transferred the PVDF membrane to 10 ml of the primary antibody reaction liquid (To 10 ml of blocking solution, we added 100 µl of ATPA monoclonal antibody to detect ATPA and 2 µl of rabbit antisera against ORF125 to detect ORF125 (M. Iwabuchi et al. Plant Molecular Biology (1999) 39:183-188)) and shook for 18 hours. We then transferred the PVDF membrane to 100 ml of TTBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.05% Tween 20) and shook for 10 minutes. We repeated this process three times to wash away excessive primary antibody liquid. We transferred the PVDF membrane to 10 mL of the secondary antibody reaction liquid (To 10 ml of blocking solution, we added goat anti-mouse IgG (manufactured by Amersham Corporation) added with 10 µl of peroxidase to detect ATPA and goat anti-rabbit IgG (manufactured by Bio-rad Corporation) added with 10 µl of alkali phosphatase to detect ORF125 (M. Iwabuchi et al. Plant Molecular Biology (1999) 39:183-188)) and shook each for one hour. Then, we transferred the PVDF membrane to 100 ml of TTBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.05% Tween 20) and shook for 10 minutes. We repeated this process three times to wash away excessive secondary antibody liquid. To detect ATPA, we used a chemical illumination system "ECL+" (manufactured by Amersham Corporation) for peroxidase and exposed for 5 minutes. To detect ORF125, we used BCIP/NBT (manufactured by Moss Inc.), a chromogenic substrate for alkali phosphatase, and developed for 5 minutes.

[0151]

As a result of comparing two lines of the buds of cytoplasmic male sterile rapeseed, fertility-restored rapeseed and the bud of rapeseed transformed by inserting the DNA into the cytoplasmic male sterile line, we found that the accumulated amount of ORF125 protein predominantly decreased in the transformed rapeseed, though there was hardly any difference in the accumulated amount of ATPA, the control. The level of a decrease was equivalent to that in the fertility-restored line formed by introducing a fertility restorer gene into the cytoplasmic male sterile line by crossbreeding (See Fig. 4 and M. Iwabuchi et al. Plant Molecular Biology (1999) 39:183-188). Furthermore, as a result of the comparison with the diluted line with regard to the level of a decrease in the accumulated amount of ORF125 protein, we found that it was  $1/8 \sim 1/16$  in the fertility-restored rapeseed and approximately  $1/8$  in the transformed rapeseed. Thus, since the restoration of fertility in rapeseed and a decrease in the accumulated amount of ORF125 protein are strongly related to the point that they are substantially equivalent, it has been proved that the present DNA sequence acts as decreasing the amount of ORF125 protein accumulated in mitochondria and is nothing but the genomic DNA sequence retaining a fertility restorer gene.

Furthermore, we took anthers from a blooming plant body to observe under a microscope and confirmed that normal pollens had been formed.

[0152]

[Effect of the Invention]

In the present invention, an Rf gene and specifically an Rf1 gene originated from radish was isolated and its structure identified. Moreover, the present invention has successfully provided a means of establishing a restored rapeseed line using the isolated Rf gene.

[0153]

[Sequence Listing]